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OPERATIONAL NOTE

EVALUATION OF A POLYMERASE CHAIN REACTION ASSAY FOR DETECTION OF PYRETHROID INSECTICIDE RESISTANCE IN THE MALARIA VECTOR SPECIES OF THE *ANOPHELES GAMBIAE* COMPLEX

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ABSTRACT. A published polymerase chain reaction assay proved reliable for detecting nerve-insensitivity (*kdr*-type) resistant and susceptible alleles to the pyrethroid insecticide permethrin in the malaria vectors *Anopheles gambiae* s.s. and *An. arabiensis* and the nonvector species *An. quadriannulatus.* The assay detects pyrethroid susceptible and resistance alleles mediated by a mutation in region II of the *para*-type sodium channel gene, showing that resistance is conferred by pyrethroid target site insensitivity. Analysis of results of the assay suggests that more than 1 mechanism conferring pyrethroid resistance is operating in an *An. gambiae* s.s. strain from Côte d'Ivoire. The value of the assay as a tool for monitoring this mode of insecticide resistance in wild populations is discussed.

KEY WORDS Pyrethroid resistance, *Anopheles gambiae* complex, polymerase chain reaction, malaria control, resistance detection

Approximately 500 million cases of malaria, resulting in an estimated 2 million deaths, are reported annually (World Health Organization 1996). The overwhelming majority of these cases occur in the Afro-tropical region, where most of the malaria vector mosquitoes belong to the *Anopheles gambiae* Giles complex. Species within this complex include the major vectors *An. gambiae* and *An. arabiensis* Patton, the minor vectors *An. merus* Dönitz, *An. melas* Theobald, and *An. bwambae* White, and the nonvector cattle-feeding member of the group *An. quadriannulatus* (Theobald) (Gillies and Coetzee 1987). It should be noted that those species listed as minor vectors are sometimes important malaria transmitters in localized areas (Temu et al. 1998). The *An. gambiae* complex has yet to be completely resolved because new members are still being discovered (Coluzzi et al. 1985, Hunt et al. 1998).

Insecticides were used for the 1st time to control adult malaria vectors in the early 1930s in South Africa (Coetzee and Hunt 1998). Extracts of the pyrethrum flowers (*Chrysanthemum*) were mixed with kerosene and sprayed inside houses with the effect of markedly reducing the incidence of malaria (Park Ross 1936). Pyrethrum was replaced in the 1940s and 1950s by organochlorine insecticides such as dichlorodiphenyltrichloroethane (DDT) and dieldrin. Public pressure arising from environmental considerations and widespread development of genetically based resistance to these insecticides by many species of insect pests led to the development and use of new classes of insecticides (Davidson 1958). Pyrethroid insecticides are now used on a large scale in agriculture and for malaria control. Pyrethroid-based control programs typically focus on the use of pyrethroid-impregnated bednets and curtains. A number of these programs have been initiated in various parts of Africa with varying degrees of success (Sexton et al. 1990, Lindsay et al. 1991, Vulule et al. 1994, Magbity et al. 1997).

Like many organochlorines, pyrethroids function as neurotoxins. The target site of DDT and pyrethroids is the voltage-dependent sodium channel of nerve axons (Bloomquist 1996). Nerve impulse conduction is eventually blocked because the insecticide prevents the sodium channel from returning to the nonconducting closed gate configuration after an action potential (Soderlund and Bloomquist 1989). One mechanism of pyrethroid resistance is conferred by alteration of the sodium channel protein to produce reduced sensitivity to DDT and pyrethroids. Martinez-Torres et al. (1998) have shown that this alteration is associated with a mutation in the *para*-type sodium channel gene of *An. gambiae.* Analysis of the domain II region of this gene from pyrethroid-susceptible (laboratory stocks) and pyrethroid-resistant (Côte d'Ivoire and Burkina Faso) strains of *An. gambiae* identified a point mutation of leucine to phenylalanine. This mutation has also been observed in resistant houseflies (*Musca domestica* L.) (Williamson et al. 1996) and German cockroaches (*Blattella germanica* (L.)) (Miyazaki et al. 1996). The similar mode of action of DDT and pyrethroids implies that the development of re-

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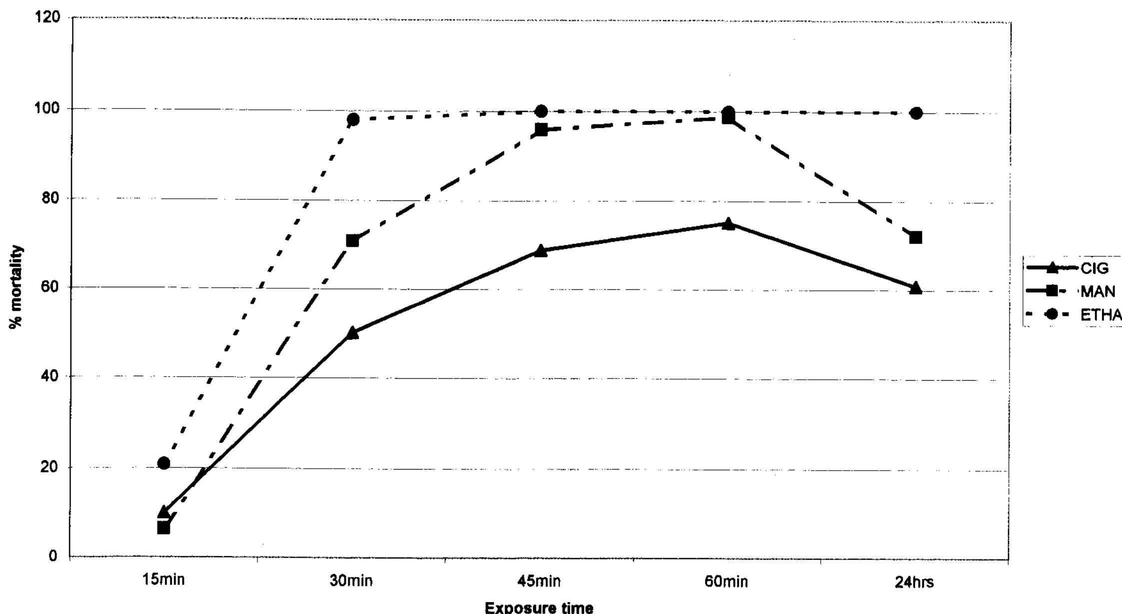

Fig. 1. Bioassay results for *Anopheles gambiae* (CIG) and *An. arabiensis* (ETHA and MAN) after exposure to 0.25% permethrin.

sistance to 1 class can confer cross-resistance to the other (Prasittisuk and Curtis 1982).

A polymerase chain reaction (PCR) assay has been developed to detect the susceptible and resistant point mutations in *An. gambiae* (Martinez-Torres et al. 1998). This assay has now been validated on a range of mosquito species held in colony at the South African Institute for Medical Research (SAIMR) in Johannesburg.

Collections of *An. gambiae* from Petionara outside Bouake, Côte d'Ivoire, were colonized in 1997 and selected for permethrin resistance. This colony (CIG) was used as a positive control for the PCR assay. Other colonies used were *An. arabiensis* MAN from Mananga, Swaziland, KGB from Kanyemba, Zimbabwe, and ETHA from Jimma, Ethiopia; *An. merus* MAF from the Kruger National Park, South Africa; and *An. quadriannulatus* SKUQUA also from the Kruger National Park. All strains are maintained in colony at the SAIMR.

Permethrin resistance was assessed using the bottle bioassay method of Brogdon and McAllister (1998) at the discriminating permethrin dosage of 0.25%. A commercial preparation of permethrin was diluted to the required concentration and used to coat 250-ml capped glass bottles. Ten microliters of 0.25% permethrin was used to coat each bottle with 1 ml of acetone as a carrier. The bottles were rotated until all the acetone had evaporated and were then left to dry for at least 3 h in a darkened cupboard. Twenty to 25 mosquitoes were aspirated into each of 4 bottles and percentage knockdown was recorded every 15 min. Each trial was replicated 3 times and results were averaged for each strain tested. The mosquitoes were removed from the exposure bottles after 1 h and overall percentage mortality was recorded after a 24-h resting period, during which time a glucose solution was made available to survivors. During selection for insecticide resistance, survivors were collected and their F_1 progeny subjected to the same procedure. Two rounds of selection for resistance to 0.25% permethrin were completed in the CIG colony in this way.

Mosquito DNA was extracted using the method of Collins et al. (1987). The diagnostic PCR assay for pyrethroid resistance was slightly modified from that of Martinez-Torres et al. (1998). Extracted genomic DNA was added to 25 μl of PCR mixture containing 2.5 μl of 10× buffer, 1 mM $MgCl_2$, 2.5 μl of 2.5 mM of each deoxynucleoside triphosphate, 0.3 mM each of primers Agd1, Agd2, Agd3, and Agd4 (Martinez-Torres et al. 1998), and 1 unit of thermostable Taq DNA polymerase. Volume was made up to 25 μl by adding double-distilled H_2O. The preparation of this mastermix was carried out on ice. The PCR reaction conditions were standardized at 94°C for 1 min, 48°C for 2 min, and 72°C for 2 min for 40 cycles with a final extension step at 72°C for 10 min. The amplified fragments were analyzed using a 2.5% agarose gel stained with ethidium bromide and visualized under ultraviolet light. Primers Agd1 and Agd2 amplify a 293-base-pair (bp) fragment common to all genotypes. With-

Table 1. Polymerase chain reaction (PCR) genotype frequencies for *Anopheles gambiae* CIG samples.[1]

PCR genotype	SS	SR	RR	Total
15-min knockdown	12	0	0	12
Survivors	3	2	7	12

[1] S, susceptible allele; R, resistance allele.

in this fragment are the 195-bp fragment amplified by Agd1 and Agd3 in resistant insects and the 137-bp fragment amplified by Agd2 and Agd4 in susceptible insects.

Permethrin resistance was 1st detected and subsequently selected for in the CIG strain of *An. gambiae* using the bottle bioassay method. Knockdown and mortality rates (Fig.1) were significantly lower in the CIG strain than in the *An. arabiensis* ETHA and MAN strains ($P < 0.0001$).

In follow-up trials, CIG mosquitoes that were knocked down after 15 min and survivors after 1 h of exposure were collected. Extractions of DNA were performed and the sodium channel genotypes were analyzed by PCR. The results are summarized in Table 1. The 15-min knockdown sample showed only susceptible alleles ($p_s = 1.0$), whereas the survivor sample had a high frequency of resistance alleles ($p_r = 0.67$) together with susceptible alleles. Genotype frequencies for the 2 groups differed significantly ($\chi^2 = 36.0$, $P < 0.0001$). All possible genotypes visualized electrophoretically are shown in Fig. 2.

Three other members of the *An. gambiae* complex were tested using the same PCR assay. Individuals from the following colonies showed amplification fragments suggesting homozygous susceptibility: SKUQUA (*An. quadriannulatus*, $n = 12$), KGB and MAN (*An. arabiensis*, $n = 20$), and MAF (*An. merus*, $n = 8$). All *An. merus* samples failed to show amplification of the 293-bp band with Agd1 and Agd2 and only 60% of those tested showed amplification for the susceptible allele with Agd2 and Agd4. Two wild-caught individuals identified as the malaria vector *Anopheles funestus* Giles from Tanzania also produced a 137-bp product but no 293-bp product, as in *An. merus*, indicating homozygous susceptibility to pyrethroids. However, in both cases target DNA product concentration was very low and both *An. funestus* specimens showed high amplification for a product of approximately 800 bp in length. No resistance alleles were seen in any of the above samples.

The nerve-insensitivity PCR assay for this mechanism of pyrethroid resistance proved reliably diagnostic for the point mutation seen in the *para*-type sodium channel gene. The technique is robust in terms of accurately determining an individual's genotype at this locus. In order to reduce primer–dimer formation and to ensure primer annealing to the target DNA, the mastermix was made on ice.

Although the primers were designed from region

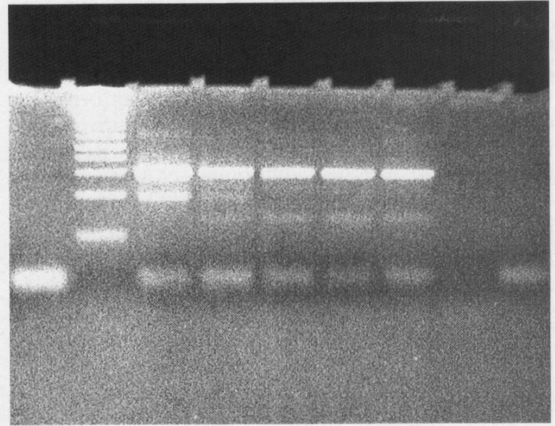

Fig. 2. Knockdown resistance (*kdr*) polymerase chain reaction assay products using 5 anopheline species. Lane 1, negative control; lane 2, 1 kbp ladder; lane 3, *Anopheles gambiae* (CIG) homozygous resistant; lane 4, *An. gambiae* (CIG) heterozygous; lane 5, *An. gambiae* (CIG) homozygous susceptible; lane 6, *An. arabiensis* (MAN) homozygous susceptible; lane 7, *An. quadriannulatus* (SKUQUA) homozygous susceptible; lane 8, *An. merus* (MAF), no detectable products; lane 9, *An. funestus,* no detectable products.

II of the *para*-type sodium channel gene of *An. gambiae,* they were diagnostic for *An. quadriannulatus* and *An. arabiensis.* This result is perhaps not surprising because one would expect this particular region of the genome to be highly conserved across a wide range of species. The absence of the 293-bp band from primers Agd1 and Agd2 in all *An. merus* and *An. funestus* specimens tested suggests that the Agd1 primer is not annealing correctly.

This assay may prove a valuable addition to the biochemical and molecular tools already developed for the detection of insecticide resistance in mosquitoes (Hemingway et al. 1987, Hemingway and Karunaratne 1998). However, other pyrethroid resistance mechanisms may already be operating in some strains. The resistance allele frequency in survivors to permethrin exposure detected by the PCR assay was only 67%. The presence of a DNA fragment indicative of homozygous susceptibility in some bioassay survivors (Table 1) suggests that another mechanism of permethrin resistance exists in the CIG colony. This finding will need to be corroborated by a more intensive investigation into the nature of additional resistance mechanisms in this colony. Preliminary biochemical investigations (Penilla et al. 1998) using unselected and selected CIG strains for permethrin resistance suggest that this mechanism may be associated with an elevated esterase (unpublished data). This possibility makes the use of both biochemical tests and standard bioassays based on published insecticide discriminat-

ing dosages essential for the detection of resistance in wild populations. Molecular and biochemical techniques should be used to reliably verify the bioassay results and can provide valuable information on resistance allele frequencies and the operational mode of insecticide resistance. The PCR assay has the advantage of being able to detect heterozygotes and because this particular mechanism of pyrethroid resistance is recessive in expression, the PCR assay is essential for accurately determining the resistance allele frequency in any wild population.

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